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THE ANTIGENIC PROPERTIES OF GLYCOPROTEINS *

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INTRODUCTION (CHEMISTRY AND SOURCES OF GLYCOPROTEINS)

Many of the recent studies in immunological reactions have had to do with the antigenic properties of compound proteins. These, for the most part, have dealt with the nucleoproteins as antigens, which subject has been thoroughly reviewed by Wells.[1] The present study deals with the antigenic properties of certain mucin substances which were prepared and used as examples of that class of compound proteins known as glycoproteins. It has been shown by Wells[2] that a mucin prepared from the gastric mucosa of pigs is capable of acting as an efficient anaphylactogen in guinea-pigs, and this fact suggested a more complete investigation of the biological reactions of the glycoproteins.

The glycoproteins are those compound proteins which yield, on cleavage, proteins and carbohydrate complexes. It is interesting to note that Abderhalden[3] has proposed dropping the name glycoproteins and would rather consider these bodies as simple proteins, because the carbohydrate group does not seem to bear the same relationship to the protein that the nucleic acid does in the nucleoproteins. Numerous chemical analyses of various mucinous substances have been made, and true mucins have been distinguished from mucoids on the basis of solubilities and precipitation properties. According to the investigations of Levene[4] and others some so-called mucins (tendon mucin) contain chondroitin-sulphuric acid or a similar substance, and should be classified as chondroproteins. Hammarsten[5] says that the mucin question has not been sufficiently studied to give any positive statements in regard to the occurrence of mucin. We know, however, that glycoproteins are found in two chief localities in the mammalian body: (1) as a product of epithelial cells; (2) in the interstices of connective tissue, especially of tendons. It is probable that the term mucin covers a number of related bodies among which differences exist with respect

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[1] Ztschr. f. Immunität., 1913, 19, p. 599.

[2] Jour. Infect. Dis., 1911, 9, p. 168.

[3] Lehrb. d. physiol. Chem., 1909, p. 191.

[4] Ztschr. f. physiol. Chem., 1901, 31, p. 395.

[5] Text Book of Physiological Chemistry (Eng. Trans. by Mandel), 1911, 7th Ed., p. 165.

to either the protein or the carbohydrate radical, or with respect to both these components.

For the purpose of determining the antigenic properties of the glycoproteins, as a class of compound proteins, three mucin preparations were made. These preparations represent both the main sources of mucin (epithelium and connective tissue) and they are extracted from the tissues of two different species (ox and pig), so that it is possible to observe not only the antigenic property of each mucin, but also the reactions of each mucin against a different type of mucin from the same species as well as against the mucins from a different species. This involves, of course, a consideration of the questions of absolute specificity of the mucins, of biological specificity against the serum of the same species and of protein specificity independent of species.

DESCRIPTION OF PREPARATIONS

The mucin substances which were selected as typical forms are (1) ox tendon mucin, (2) ox submaxillary mucin, (3) swine stomach mucin. They were prepared and purified as well as possible according to methods which have been used by various investigators for the purpose of chemical analysis. There is some question, as there always must be with our present methods of preparation of proteins, whether each mucin preparation is a chemical individuality; and it is not certain that all other proteins were separated from the protein component of the mucin combination. The preparations represent products which have been regarded as pure mucins and for our present purpose we shall consider them as such. In their dry state they appear as white or yellowish gray powders. They are almost altogether insoluble in water but can be dissolved in large amounts of weak alkali. They give the color reactions of the proteins (biuret, Millon, Hopkins-Cole). When heated for a long time on the water bath with 2 percent hydrochloric acid or 5 percent sulphuric acid, the solution becomes dark brown. After this is cooled and made alkaline with solid potassium hydroxid, a test by Fehling's solution shows a reduction. The principle involved in the preparation of mucins is a precipitation by means of acid from water or dilute alkali extracts of the tissue. Acetic acid is used in most methods which are described in the literature for separating mucinous substances from different sources.[6] Hammarsten has shown that precipitation by acetic acid cannot be used in the preparation of mucin from the submaxillary gland, as another protein substance is precipitated with the mucin, but this substance remains in solution if hydrochloric acid is used. Moreover, it seems best to use hydrochloric acid in all preparations of mucins since Posner and Gies[7] have shown that when mucoid substances are precipitated with acid from tissue extracts, non-mucoid material is withdrawn from the fluid in combination with the mucoid. If more non-mucoid material remains in solution when hydrochloric acid is used than when acetic acid is used, it is plain that in seeking for products of relatively high purity the ordinary method of precipitating with acetic acid should not be used. Accordingly, all precipitations have been made with hydrochloric acid. In the preparation of ox tendon mucin the method of Chittenden and Gies[8] was used. Ox submaxillary mucin was prepared after the method of Hammarsten.[9] Swine stomach mucin seems to have been prepared usually from an extract of the stomach epithelium by precipitation with acetic acid,[10] or by scraping off the crude mucinous secretion from a number of stomachs taken from fasting pigs, and purifying this by means of solution in dilute alkali and precipitation with acetic acid.[11] The swine stomach mucin used in the present study, however, was precipitated from an extract of the stomach linings by means of hydrochloric acid. Detailed descriptions of the methods used for the mucin preparations are as follows:

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[6] Abderhalden, Muzinsubstanzen, in Handbk. der Biochem. Arbeitsmeth., 1911; also Mucin in Biochem. Handlex.

[7] Amer. Jour. Physiol., 1904, 11, p. 404.

Ox tendon mucin.—Fresh Achilles tendons of oxen were freed as carefully as possible from all adherent tissues, cut into small pieces and ground in a meat grinder. The tissue was then washed in distilled water for twenty-four hours — one liter of water being used for every 500 grams. After the water was removed the tissue was washed for twenty-four hours in the same amount of 10 percent salt solution. The salt solution was then decanted and the tissue washed with running water until the chlorid was entirely removed. The tissue was next pressed as dry as possible and extracted for sixty-five hours with half saturated limewater (one liter for every 500 grams). A second extraction of forty-eight hours was also made. From these extracts, which were filtered through paper, the mucin was precipitated by the addition of large amounts of 0.2 percent hydrochloric acid. The precipitate was purified in the following manner: It was dissolved in as small an amount of 0.5 percent sodium carbonate as possible. This solution was filtered, made nearly neutral by the addition of a little 10 percent hydrochloric acid, and then precipitated by 0.2 percent hydrochloric acid. The process was then repeated. The precipitate was next washed thoroughly with a large amount of 0.2 percent hydrochloric acid, and lastly with water until the acid was entirely removed. It was then transferred to 50 percent alcohol, next to 95 percent alcohol, then to absolute alcohol, and finally to alcohol-ether in which the mucin was allowed to stand for some time with frequent changes. Dried in a vacuum desiccator over sulphuric acid, the preparation was easily reduced to a powder.

Ox submaxillary mucin.—Fresh ox submaxillary glands, after being cut as free as possible from fat and surrounding tissues, were cut up very finely by means of a meat grinder. The tissue was extracted with distilled water (one liter for every 500 grams) for thirty-six hours. This watery extract was filtered through a heavy filter with the aid of suction, and then treated with 10 percent hydrochloric acid until the strength of the liquid was 0.15 percent hydrochloric acid. On addition of the acid the mucin was precipitated, but it dissolved upon stirring. Immediately four volumes of distilled water were added and the mucin separated as a slimy mass, which could be picked up easily on a bent stirring rod. It was purified by dissolving in 0.15 percent hydrochloric acid, filtering rapidly and immediately reprecipitating by diluting with water. This process was repeated and then the mucin was thoroughly washed with water, transferred to alcohol, treated with alcohol-ether, dried, and reduced to a powder form in the same manner as described above.

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[8] Jour. Exper. Med., 1896, 1, p. 186.

[9] Ztschr. f. physiol. Chem., 1888, 12, p. 163.

[10] Abderhalden, Biochem. Handlex., p. 140.

[11] Lopez-Suarez, Biochem. Ztschr., 1913, 56, p. 167.

TABLE 1
ANAPHYLAXIS EXPERIMENTS WITH OX TENDON MUCIN

Guinea-Pig	Second Injection	Symptoms	Days Interval	Third Injection	Symptoms	Days Interval	Fourth Injection	Symptoms
17	0.05 gm. ox tendon mucin	Severe with death in 1 hour and 45 minutes	..	....	....	..	....	....
18	0.05 gm. ox tendon mucin	Moderate	19	0.05 gm. ox tendon mucin	Doubtful	..	....	....
28	0.05 gm. ox submax. mucin	None visible; slight drop in temperature	19	0.05 gm. ox submax. mucin	None visible; slight drop in temperature	2	0.05 gm. ox tendon mucin	Moderate
30	0.05 gm. ox submax. mucin	Doubtful	..	....	....	..	....	....
16	0.05 gm. pig stomach mucin	None	19	0.05 gm. swine stomach mucin	None visible; slight drop in temperature	2	0.05 gm. ox tendon mucin	Severe
19	0.05 gm. pig stomach mucin	None	19	0.05 gm. swine stomach mucin	None visible; drop in temperature	2	0.05 gm. ox tendon mucin	Severe
29	1 c.c. ox serum	Severe with death in 1 hour	..	....	....	..	....	....
31	1 c.c. ox serum	Moderate	7	0.5 c.c. ox serum	Slight	2	0.05 gm. ox tendon mucin	Slight

Each guinea-pig received a sensitizing dose of 0.005 gm. of ox tendon mucin. Before the second injection was given an interval of at least 21 days had elapsed.

Swine stomach mucin.—Fresh swine stomach linings (the stripped mucosa obtained from the Pepsin Department of Armour & Co.) were finely chopped up by means of a meat grinder and then extracted with water for twenty-four hours (using one liter of water for every 500 grams). When the water extract was removed, the tissue was pressed as dry as possible and then extracted with the same amount of 10 percent salt solution for twenty-four hours. When the salt solution extract was removed, running water was allowed to pass through the tissue until all the sodium chlorid was removed and then an extract was made with half-saturated lime water for twenty-four hours (using one liter for every 500 grams). The lime water extract was then filtered through paper and, when about one-fourth a volume of 0.2 percent hydrochloric acid was added, the mucin precipitated. This product was collected and purified by redissolving it in 0.5 percent sodium carbonate, filtering, adding 10 percent hydrochloric acid to the neutral point, and then reprecipitating the mucin by adding an excess of 0.2 percent hydrochloric acid. This process was repeated twice. A centrifuge was used to throw down the precipitate. Finally, the mucin was washed free from acid with distilled water, treated with alcohol and alcohol-ether, and dried in the same manner as the other preparations.

In all cases the extraction was done at low temperatures and whenever it was necessary for a solution or a precipitate to stand for some time a little chloroform was added to guard against putrefaction.

THE ANAPHYLAXIS REACTIONS

Mucin does not possess a "primary" toxicity for animals into which it is injected. Wells[12] states that he has injected rabbits with large amounts of pure ox tendon mucin almost daily for two to four months without observing any ill effects. It has been mentioned that Wells has also demonstrated that pig stomach mucin is an efficient anaphylactogen; i. e., it will produce sensitization which, after a certain time, may be followed by intoxication of susceptible animals. The specificity of the anaphylaxis reaction and its value as a special biochemical method of study are well established facts and do not need to be dwelt upon here.

A few statements regarding the method of conducting experiments and describing results are perhaps necessary. Guinea-pigs of medium weight (averaging about 300 gm.) were used. The mucins were dissolved in 0.2 percent sodium hydroxid and the solution diluted with an equal volume of water before injection, making a 0.1 percent sodium hydroxid solution. A sensitizing dose of 0.005 gm. and an intoxicating dose of 0.05 gm. were used in all the experiments. In the case of serum sensitization and intoxication the doses used were 0.1 c.c. and 1 c.c. All injections were made into the peritoneal cavity. The usual special precautions regarding the thorough cleansing of syringes, glass utensils, and needles were adopted in order to avoid contamination of the injected proteins with foreign proteins. In recording the effects produced by the second or intoxicating dose as well as all subse-

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[12] Chemical Pathology, 1914, 2d Ed., p. 389.

TABLE 2

ANAPHYLAXIS EXPERIMENTS WITH OX SUBMAXILLARY MUCIN

Guinea-Pig	Second Injection	Symptoms	Days Interval	Third Injection	Symptoms	Days Interval	Fourth Injection	Symptoms
8	0.05 gm. ox submax. mucin	Severe with death in 37 minutes	..	....	....	..	....	....
9	0.05 gm. ox submax. mucin	Severe	..	0.05 gm. ox submax. mucin	....	..	....	....
10	0.05 gm. ox tendon mucin	None	2	....	Severe	..	....	....
11	0.05 gm. ox tendon mucin	None	..	....	....	..	....	....
13	0.05 gm. pig stomach mucin	None	19	0.05 gm. swine stomach mucin	None visible; drop in temperature	..	....	....
14	0.05 gm. pig stomach mucin	Doubtful	19	0.05 gm. swine stomach mucin	None	..	....	....
12	1 c.c. ox serum	Moderate	7	0.5 c.c. ox serum	None	2	0.05 gm. ox submax. mucin	Moderate
15	1 c.c. ox serum	Slight	..	....	....	..	....	....

Each guinea-pig received a sensitizing dose of 0.005 gm. of ox submaxillary mucin. Before the second injection was given an interval of at least 21 days had elapsed.

TABLE 3

ANAPHYLAXIS EXPERIMENTS WITH PIG STOMACH MUCIN

Guinea-Pig	Second Injection	Symptoms	Days Interval	Third Injection	Symptoms	Days Interval	Fourth Injection	Symptoms
21	0.05 gm. pig stomach mucin	Moderate	..	....	....	..	....	....
23	0.05 gm. pig stomach mucin	Moderate	..	....	....	..	....	....
20	0.05 gm. ox tendon mucin	None	19	0.05 gm. ox tendon mucin	None visible; slight drop in temperature	2	0.05 gm. pig stomach mucin	Severe with death in 18 hours
22	0.05 gm. ox tendon mucin	None	..	....	....	..	....	....
24	0.05 gm. ox submax. mucin	None	19	0.05 gm. ox submax. mucin	Marked drop in temperature	2	0.05 gm. pig stomach mucin	None
27	0.05 gm. ox submax. mucin	Slight	19	0.05 gm. ox submax. mucin	Severe with death in 1 hour and 4 minutes	..	....	....
25	1 c.c. pig serum	Severe with death in 20 minutes*	..	....	....	..	....	....
26	1 c.c. pig serum	Severe	..	....	....	..	....	....

Each guinea-pig received a sensitizing dose of 0.005 gm. of pig stomach mucin. Before the second injection was given an interval of at least 21 days had elapsed.

*This pig found to be in a late stage of pregnancy at autopsy.

quent doses, the following terms were used with the constant significance, as given by Wells:[13]

"1.—'Doubtful' symptoms. The animal scratches itself a few times and perhaps seems a little uneasy or a trifle ill; the temperature remains normal or falls not more than 1-1.5 C. Such results may be occasionally observed after injection of foreign proteins into non-sensitized animals, and are not regarded as of any significance.

"2.—'Slight' symptoms. The animal does not become seriously ill, but scratches itself vigorously, is either very restless or very somnolent or both alternately, hair roughened, eyes usually lachrymate and partly closed. The temperature usually falls 1-1.5 C. Such a reaction is believed to indicate usually, if not always, a real sensitization and a true anaphylactic intoxication, but it may possibly sometimes, but rarely, occur from foreign proteins without sensitization.

"3.—'Moderate' symptoms. More seriously ill than under 2. Usually the hair becomes very rough, with respiratory distress and marked lachrymation, fall of temperature of 1.5-3.5 C.; frequently the animal lies down and sometimes there is violent coughing.

"4.—'Severe' symptoms. Same as under 3, but so much more severe as to threaten to cause death, but followed by complete recovery in a few hours. In these animals the temperature usually falls 3 to 4 degrees or even more."

TABLE 4
ANAPHYLAXIS EXPERIMENTS WITH OX SERUM

Guinea-Pig	Second Injection	Symptoms	Days Interval	Third Injection	Symptoms
1	0.05 gm. ox tendon mucin	None	..	....	....
5	0.05 gm. ox tendon mucin	Doubtful	2	0.5 c.c. ox serum	Moderate
7	0.05 gm. ox tendon mucin	Slight	3	0.5 c.c. ox serum	Moderate
2	0.05 gm. ox sub-. max. mucin	Doubtful	3	0.5 c.c. ox serum	Severe
3	0.05 gm. ox submax. mucin	Slight	2	0.5 c.c. ox serum	Severe
4	0.05 gm. ox submax. mucin	Slight	..	....	....

Each guinea-pig received a sensitizing dose of 0.1 c.c. of ox serum. Before the second injection was given an interval of at least 21 days had elapsed.

TABLE 5
ANAPHYLAXIS EXPERIMENTS WITH SWINE SERUM

Guinea-Pig	Second Injection	Symptoms	Days Interval	Third Injection	Symptoms
32	0.05 gm. swine stomach mucin	Doubtful	5	0.5 c.c. swine serum	Moderate
33	0.05 gm. swine stomach mucin	Slight	5	0.5 c.c. swine serum	Slight

Each guinea-pig received a sensitizing dose of 0.1 c.c. of pig serum. Before the second injection was given an interval of at least 21 days had elapsed.

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[13] Jour. Infect. Dis., 1911, 8, p. 88.

These experiments show that with each mucin it was possible to produce anaphylactic intoxication in sensitized guinea-pigs, the reaction varying in degree from moderate symptoms to death. About the same reactions were obtained in guinea-pigs sensitized with the mucin when an intoxicating dose of blood-serum from the same species was given. The evidence is very slight that sensitization occurred when a guinea-pig was injected with one mucin preparation so that it would react positively upon receiving an intoxicating dose of the other mucin preparations from the same species as well as from a different species. However, a comparison of the records for Guinea-pigs 28 and 16 (Table 1) shows that, when subsequent injections were made for the purpose of determining the presence of a refractory period, the anti-anaphylactic condition was greater in the case of ox tendon mucin against ox submaxillary mucin than it was in the case of ox tendon mucin against pig stomach mucin. In these cases, while the symptoms following the third injection noted are "none visible," yet a drop in temperature was observed which alone might be taken to indicate a positive reaction; such a reaction can easily be accounted for by a sensitization with the second injection, as there was an interval of nineteen days between the injections. All the animals sensitized with either ox serum or pig serum did not react positively, or, at the most, only doubtfully or slightly so, to the mucins from the same species in each case, and the mucins did not render them refractory to the sera. On the basis of these experiments it might be interpreted that some traces of blood proteins were present in the mucin preparations.

THE PRECIPITIN REACTIONS

In order to study the precipitin reactions, young full-grown male rabbits were selected for the production of immune sera. The method first advocated by Fornet and Müller[14] was used. These investigators and others[15] have shown that when rabbits are given intraperitoneal injections of increasing amounts of foreign serum on three successive days there results a considerable production of precipitins. Hektoen[16] has obtained the same results and he shows by charts that the acme of antibody content in the serum is reached about the twelfth day after the last injection. The doses of serum used are usually 5, 10, and 15 c.c.

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[14] Ztschr. f. biol. Technik u. Methodik, 1908, 1, p. 201

[15] Bonhoff and Tsuzuki, Ztschr. f. Immunitätsf., Orig., 1910, 4, p .180. Gay and Fitzgerald, Univ. Cal. Publ. in Path., 1912, 2, p. 77.

[16] Jour. Infect. Dis., 1914, 14, p. 403.

TABLE 6
PRECIPITIN TEST WITH OX TENDON MUCIN

Dilution of Antigen 1 Percent Solution	Ox Tendon Mucin Antiserum	Normal Rabbit Serum	Ox Submax. Mucin Antiserum	Swine Stomach Mucin Antiserum	Ox Serum Antiserum	Swine Serum Antiserum	Egg Albumin Antiserum	No Antiserum
0.5 c.c.	Turbid and ppt.	Clear	Trace ppt.	Trace ppt.	Turbid	Clear	Clear	Clear
0.25 c.c.	Ppt.	Clear	Trace ppt.	Slight ppt.	Trace ppt.	Clear	Clear	Clear
0.125 c.c.	Ppt.	Clear	Clear	Trace ppt.	Slight ppt.	Clear	Clear	Clear
0.0625 c.c.	Ppt.	Clear	Trace ppt.	Trace ppt.	Trace ppt.	Clear	Clear	Clear
0.03125 c.c.	Slight ppt.	Clear	Trace ppt.	Trace ppt. (?)	Trace ppt.	Clear	Clear	Clear
0.0156 c.c.	Trace ppt.	Clear	Turbid	Clear	Turbid	Clear	Clear	Clear
0.0078 c.c.	Trace ppt.	Clear	Turbid	Clear	Trace ppt.	Clear	Clear	Clear
0.0039 c.c.	Trace ppt.	Clear	Clear	Clear	Clear	Clear	Clear	Clear
0.0019 c.c.	Trace ppt.	Clear	Clear	Clear	Clear	Clear	Clear	Clear
0.0009 c.c.	Trace ppt.	Clear	Clear	Clear	Clear	Clear	Clear	Clear
No antigen	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear

TABLE 7
PRECIPITIN TEST WITH OX SUBMAXILLARY MUCIN

Dilution of Antigen 1 Percent Solution	Ox Submax. Mucin Antiserum	Ox Submax. Mucin Antiserum	Normal Rabbit Serum	Ox Tendon Mucin Antiserum	Swine Stomach Mucin Antiserum	Ox Serum Antiserum	Swine Serum Antiserum	Egg Albumin Antiserum
0.5 c.c.	Turbid	Turbid and ppt.	Slightly turbid	Turbid	Clear	Turbid	Turbid	Slight ppt.
0.25 c.c.	Turbid	Turbid	Clear	Slight ppt.	Clear	Slight ppt.	Slightly turbid	Turbid
0.125 c.c.	Ppt.	Ppt.	Clear	Slight ppt.	Clear	Slight ppt.	Clear	Clear
0.0625 c.c.	Ppt.	Ppt.	Clear	Slight ppt.	Clear	Turbid	Clear	Clear
0.03125 c.c.	Ppt.	Slight ppt.	Clear	Clear	Clear	Clear	Clear	Clear
0.0156 c.c.	Slight ppt.	Slight ppt.	Clear	Clear	Clear	Clear	Clear	Clear
0.0078 c.c.	Slight ppt.	Slight ppt.	Clear	Clear	Clear	Clear	Clear	Clear
0.0039 c.c.	Slight ppt.	Slight ppt.	Clear	Clear	Clear	Clear	Clear	Clear
0.0019 c.c.	Trace ppt.	Trace ppt.	Clear	Clear	Clear	Clear	Clear	Clear
0.0009 c.c.	Trace ppt. (?)	Clear	Clear	Clear	Clear	Clear	Clear	Clear
No antigen	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear

TABLE 8
PRECIPITIN TEST WITH PIG STOMACH MUCIN

Dilution of Antigen 1 Percent Solution	SwineStomach Mucin Antiserum	SwineStomach Mucin Antiserum	Normal Rabbit Serum	Ox Tendon Mucin Antiserum	Ox Submax. Mucin Antiserum	Ox Serum Antiserum	Swine Serum Antiserum	Egg Albumin Antiserum
0.5 c.c.	Turbid and Slight ppt.	Turbid	Clear	Trace ppt.	Trace ppt.	Clear	Ppt.	Clear
0.25 c.c.	Ppt.	Turbid	Clear	Trace ppt.	Trace ppt.	Clear	Ppt.	Clear
0.125 c.c.	Ppt.	Slight ppt.	Clear	Clear	Clear	Clear	Slight ppt.	Clear
0.0625 c.c.	Ppt.	Slight ppt.	Clear	Clear	Clear	Clear	Slight ppt.	Clear
0.03125 c.c.	Slight ppt.	Ppt.	Clear	Clear	Clear	Clear	Slight ppt.	Clear
0.0156 c.c.	Slight ppt.	Slight ppt.	Clear	Clear	Clear	Clear	Trace ppt.	Clear
0.0078 c.c.	Slight ppt.	Slight ppt.	Clear	Clear	Clear	Clear	Clear	Clear
0.0039 c.c.	Trace ppt.	Trace ppt.	Clear	Clear	Clear	Clear	Clear	Clear
0.0019 c.c.	Trace ppt.	Clear	Clear	Clear	Clear	Clear	Clear	Clear
0.0009 c.c.	Trace ppt. (?)	Clear	Clear	Clear	Clear	Clear	Clear	Clear
No antigen	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear

injected one day apart. Accordingly, the preparation of the immune sera used in the present study was carried out as follows:

Rabbits were injected intraperitoneally with ox tendon mucin, ox submaxillary mucin, pig stomach mucin, ox serum, swine serum, and crystallized egg albumin. The doses of mucin used were 0.5, 1, and 1.5 gm. which were dissolved in as small amounts of 0.1 percent sodium hydroxid as possible. The same amounts of crystallized egg albumin were dissolved in water. The sera were injected in doses of 5, 10, and 15 c.c. In all cases, the triple injections were given one day apart. On the twelfth day (in two instances it was the fourteenth) after the last injection, the animals were bled to death from the carotid under ether anesthesia, the blood was allowed to clot and the serum was pipetted off and hermetically sealed in tubes. These were kept in the refrigerator until they could be used in making the tests. The crystallized egg albumin was used to produce an antiserum which served as a control in that it represented the antibody of a pure protein which is known to act as a powerful antigen. Inasmuch as it was necessary to dissolve the mucin preparations in 0.1 percent sodium hydroxid before the antigenic dilutions could be made for each test, the egg albumin was also used to control the matter of whether or not the small amounts of alkali interfered with the precipitin reaction, by making antigenic mixtures for the egg albumin precipitin test with both 0.1 percent sodium hydroxid and water. Normal rabbit serum (for control) and antiserum were always used in 0.1 c.c. amounts; the several antigens in variable dilutions made up to 0.5 c.c. The antigen mixture in the case of the mucins was a 1 percent solution in 0.1 percent sodium hydroxid with a total volume of 0.5 c.c., all dilutions being made with 0.85 percent salt solution. Observations were taken after two hours' incubation at 37 C. and again after a night's stand in the ice-box. The results of the tests are given in the following tables.

TABLE 9

PRECIPITIN TEST WITH OX SERUM

Dilution of Antigen	Ox Serum Antiserum
0.05 c.c.	Marked ppt.
0.025 c.c.	Marked ppt.
0.0125 c.c.	Marked ppt.
0.00625 c.c.	Ppt.
0.003125 c.c.	Turbid
0.00156 c.c.	Ppt.
0.00078 c.c.	Slight ppt.
0.00039 c.c.	Slight ppt.
0.00019 c.c.	Slight ppt.
0.00009 c.c.	Trace ppt.
0.000045 c.c.	Trace ppt. (?)
No antigen	Clear

No precipitate with normal rabbit serum, ox tendon mucin antiserum, ox submaxillary mucin antiserum, pig stomach mucin antiserum, pig serum antiserum, egg albumin antiserum, and no antiserum.

TABLE 10

PRECIPITIN TEST WITH PIG SERUM

Dilution of Antigen	Pig Serum Antiserum
0.05 c.c.	Turbid
0.025 c.c.	Turbid
0.0125 c.c.	Turbid
0.00625 c.c.	Ppt.
0.003125 c.c.	Ppt.
0.00156 c.c.	Slight ppt.
0.00078 c.c.	Slight ppt.
0.00039 c.c.	Slight ppt.
0.00019 c.c.	Trace ppt.
0.00009 c.c.	Clear
0.000045 c.c.	Clear
No antigen	Clear

No precipitate with normal rabbit serum, ox tendon mucin antiserum, ox submaxillary mucin antiserum, pig stomach mucin antiserum, ox serum antiserum, egg albumin antiserum and no antiserum.

While the anaphylaxis experiments demonstrated the antigenic properties of the mucins and indicated a certain specificity, the precipitin tests revealed the same facts much more clearly on account of the quantitative nature of the reactions. Each mucin produced antibodies which reacted with itself in comparatively high dilutions, but did not seem to be as powerful an antigen as the pure simple protein (crystallized egg albumin) which was tested under the same conditions as a control. Table 6 shows that the antiserum obtained from injecting ox tendon mucin into a rabbit reacts fairly well with that glycoprotein; it reacts less markedly with another glycoprotein from the same species, namely, ox submaxillary mucin; and it reacts definitely, but in a still smaller degree, with a mucin from a different species, viz., swine stomach mucin. It also reacts with the blood serum of the same species, the ox, in just as high dilutions as it reacts with either of the heterologous mucins, but it does not react at all with the blood serum of the different species, the swine. The same quantitative differences seem to hold for all three of the mucin preparations. While it was possible to interpret some of the results obtained from the anaphylaxis experiments as being due to traces of blood proteins in the mucin preparations, such an interpretation can hardly be placed upon the precipitin tests. It need scarcely be repeated here that great care was exercised in making the mucin preparations and that these products, representing glycoproteins from two sources (connective tissue and epithelium) taken from two species (ox and swine) all possessed practically the same physical properties and all gave the ordinary protein color reactions as well as yielding a carbohydrate radical on cleavage, yet in their antigenic properties they exhibited a quantitative protein specificity independent of source and species. Table 7 shows no precipitation when swine stomach mucin antiserum was added to solutions containing ox submaxillary mucin, but it is likely that in this case the test was not delicate enough to detect an antigen-antibody reaction, as the following complement-fixation tests would seem to indicate. If there is a species specificity among these glycoproteins it is very much less apparent than the protein specificity. However, in all cases, the reaction of the mucin used in immunization is produced to a greater degree with the antiserum of another mucin from the same species, than it is with the antiserum of a mucin from a different species. These results would seem to support the view that specificity depends on the chemical composition of the antigenic protein, rather than its biological origin. But as

TABLE 11

PRECIPITIN TEST WITH CRYSTALLIZED EGG ALBUMIN

Dilution of Antigen 1 Percent Solution	Egg Albumin Antiserum NaOH Solution of Antigen	Egg Albumin Antiserum H_2O Solution of Antigen	Normal Rabbit Serum NaOH Solution of Antigen	Ox Tendon Mucin Antiserum NaOH Solution of Antigen	Ox Submax. Mucin Antiserum H_2O Solution of Antigen	Pig Stomach Mucin Antiserum H_2O Solution of Antigen	Ox Serum Antiserum NaOH Solution of Antigen	No Antiserum H_2O Solution of Antigen
0.5 c.c.	Slight ppt.	Ppt.	Clear	Slightly turbid	Slightly turbid	Slightly turbid	Clear	Clear
0.25 c.c.	Slight ppt.	Ppt.	Clear	Slightly turbid	Slightly turbid	Slightly turbid	Clear	Clear
0.125 c.c.	Ppt.	Slight ppt.	Clear	Slightly turbid	Slightly turbid	Slightly turbid	Clear	Clear
0.0625 c.c.	Ppt.	Ppt.	Clear	Slightly turbid	Slightly turbid	Trace turbid	Clear	Clear
0.03125 c.c.	Ppt.	Slight ppt.	Clear	Clear	Slightly turbid	Trace turbid	Clear	Clear
0.0156 c.c.	Ppt.	Slight ppt.	Clear	Clear	Slightly turbid	Slightly turbid	Clear	Clear
0.0078 c.c.	Ppt. and turbid	Ppt. and turbid	Clear	Clear	Slightly turbid	Trace turbid	Clear	Clear
0.0039 c.c.	Ppt. and turbid	Ppt. and turbid	Clear	Clear	Slightly turbid	Trace turbid	Clear	Clear
0.0019 c.c.	Ppt. and turbid	Slight ppt.	Clear	Clear	Slightly turbid	Clear	Clear	Clear
0.0009 c.c.	Turbid	Slight ppt.	Clear	Clear	Slightly turbid	Clear	Clear	Clear
No antigen	Clear	Clear	Clear	Clear	Slightly turbid	Clear	Clear	Clear

Wells[17] has said, "Whether the chemical differences that determine specificity are of quantitative nature, which can be disclosed by analytic means, or whether they are sometimes dependent upon spatial relationships of the amino-acid radicals, as Pick suggests, remains to be determined."

THE COMPLEMENT-FIXATION REACTIONS

The complement-fixation reaction was found a more delicate test for antibodies than the precipitin reaction, as is usually the case. The tests were made with the same amounts of antigen and immune serum that were used in the precipitin reactions. The details of the technic follow:[18]

TABLE 12

COMPLEMENT-FIXATION TEST WITH OX TENDON MUCIN

Dilution of Antigen 1 Percent Solution	Ox Tendon Mucin Antiserum	Normal Rabbit Serum	Ox Submax. Mucin Antiserum	Swine Stomach Mucin Antiserum	Ox Serum Antiserum	Swine Serum Antiserum	Egg Albumin Antiserum
0.5 c.c.	—	+	Partial +	+	—	Complete hemolysis	
0.25 c.c.	—	+	Partial +	+	—		
0.125 c.c.	—	+	Partial +	+	—		
0.0625 c.c.	—	+	Partial +	+	—		
0.03125 c.c.	—	+	+	+	Trace +		
0.0156 c.c.	—	+	+	+	Trace +		
0.0078 c.c.	—	+	+	+	Trace +		
0.0039 c.c.	—	+	+	+	Trace +		
0.00195 c.c.	—	+	+	+	Trace +		
0.00097 c.c.	—	+	+	+	Trace +		
0.00048 c.c.	Trace +	+	+	+	Partial +		
0.00024 c.c.	Trace +	+	+	+	Partial +		
0.00012 c.c.	Trace +	+	+	+	Partial +		
0.00006 c.c.	Trace +	+	+	+	+		
0.00003 c.c.	Partial +	+	+	+	+		
No antigen	+	+	+	+	+		

Antigenic mixtures.—One percent solution in 0.1 percent sodium hydroxid with a total volume of 0.5 c.c. Serum used for antigen was heated (56 C. for thirty minutes).

Antiserum.—0.1 c.c. (56 C. for thirty minutes).

Complement.—0.05 c.c. of mixed serum from two guinea-pigs. This was never more than twenty-four hours old. Dilution to 0.1 c.c. in salt solution. Incubation with antigen and antibody at 37 C. for one hour.

Hemolytic system.—1 c.c. of a 5 percent suspension of washed sheep corpuscles containing two minimal hemolytic doses of a strong rabbit anti-sheep serum (1 c.c. of 1:1,000).

Positive Fixation.—Complete absence of hemolysis after two hours at 37 C. followed by sedimentation over night in the ice-box.

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17. Chemical Pathology, 1914, 2d Ed., p. 187.
18. Gay and Robertson, Jour. Exper. Med., 1912, 16, p. 476.

Controls.—Each antigenic dilution plus 0.1 c.c. of inactivated normal rabbit serum. Antiserum with salt solution replacing antigen. These should hemolyse completely.

The results of the tests are recorded in the terms of degrees of hemolysis as follows: + = complete hemolysis; — = no hemolysis; trace + = trace of hemolysis; partial + = partial hemolysis, fifty percent or more; almost + = almost complete hemolysis.

These results show specific antigen-antibody reactions in higher dilutions than observed in the precipitin tests. On the whole, the quantitative nature of the reactions is the same. In the few instances where the results of the precipitin and complement-fixation tests do not harmonize, the lack of agreement may be accounted for by the difference in delicacy of the reactions or some slight error in manipulation. Sufficient amounts of the antisera were not on hand to repeat the tests.

TABLE 13

COMPLEMENT-FIXATION TEST WITH OX SUBMAXILLARY MUCIN

Dilution of Antigen 1 Percent Solution	Ox Submax. Mucin Antiserum	Normal Rabbit Serum	Ox Tendon Mucin Antiserum	Swine Stomach Mucin Antiserum	Ox Serum Antiserum	Swine Serum Antiserum	Egg Albumin Antiserum
0.5 c.c.	—	+	Trace +	Trace +	Trace +	Complete hemolysis	
0.25 c.c.	—	+	Trace +	Trace +	Trace +		
0.125 c.c.	—	+	Trace +	Partial +	Trace +		
0.0625 c.c.	—	+	Trace +	Partial +	Trace +		
0.03125 c.c.	—	+	Partial +	Partial +	Partial +		
0.0156 c.c.	—	+	Partial +	Partial +	Partial +		
0.0078 c.c.	—	+	Partial +	Partial +	Partial +		
0.0039 c.c.	—	+	Partial +	Partial +	Almost +		
0.00195 c.c.	Trace +	+	Partial +	Partial +	Almost +		
0.00097 c.c.	Trace +	+	Partial +	Partial +	Almost +		
0.00048 c.c.	Partial +	+	Partial +	+	Almost +		
0.00024 c.c.	Partial +	+	Partial +	+	Almost +		
0.00012 c.c.	Partial +	+	Almost +	+	Almost +		
0.00006 c.c.	+	+	+	+	Almost +		
0.00003 c.c.	+	+	+	+	Almost +		
No antigen	+	+	+	+	+		

SUMMARY

A study of the results obtained from anaphylaxis experiments, as well as precipitin and complement-fixation tests, leads to the following conclusions in respect to the antigenic properties of the glycoproteins, ox tendon mucin, ox submaxillary mucin, and swine stomach mucin, which preparations were made and purified as well as possible according to standard methods.

The glycoproteins are capable of acting as antigens, but are not as powerful in their antigenic properties as simple proteins. This is in accordance with what other investigators have found to be true of

TABLE 14

COMPLEMENT-FIXATION TEST WITH SWINE STOMACH MUCIN

Dilution of Antigen 1 Percent Solution	SwineStomach Mucin Antiserum	Normal Rabbit Serum	Ox Tendon Mucin Antiserum	Ox Submax. Mucin Antiserum	Ox Serum Antiserum	Swine Serum Antiserum	Egg Albumin Antiserum
0.5 c.c.	—	+	—	Trace +	+	Trace +	+
0.25 c.c.	—	+	—	Trace +	+	Trace +	+
0.125 c.c.	—	+	Trace +	Trace +	+	Trace +	+
0.0625 c.c.	—	+	Trace +	Slight +	+	Partial +	+
0.03125 c.c.	—	+	Partial +	+	+	Partial +	+
0.0156 c.c.	—	+	+	+	+	Almost +	+
0.0078 c.c.	—	+	+	+	+	Almost +	+
0.0039 c.c.	—	+	+	+	+	Almost +	+
0.00195 c.c.	—	+	+	+	+	Almost +	+
0.00097 c.c.	—	+	+	+	+	Almost +	+
0.00048 c.c.	Partial +	+	+	+	+	Almost +	+
0.00024 c.c.	+	+	+	+	+	Almost +	+
0.00012 c.c.	+	+	+	+	+	Almost +	+
0.00006 c.c.	+	+	+	+	+	Almost +	+
0.00003 c.c.	+	+	+	+	+	Almost +	+
No antigen	+	+	+	+	+	+	+

TABLE 15

COMPLEMENT-FIXATION TEST WITH OX SERUM

Dilution of Antigen	Ox Serum Antiserum
0.05 c.c.	Trace +
0.025 c.c.	—
0.0125 c.c.	—
0.00625 c.c.	—
0.003125 c.c.	—
0.00156 c.c.	—
0.00078 c.c.	—
0.00039 c.c.	—
0.000195 c.c.	—
0.000097 c.c.	—
0.000048 c.c.	Trace +
0.000024 c.c.	Trace +
0.000012 c.c.	Partial +
0.000006 c.c.	Partial +
0.000003 c.c.	Almost +
No antigen	+

Complete hemolysis with normal rabbit serum, ox tendon mucin antiserum, ox submaxillary mucin antiserum, swine stomach mucin antiserum, swine serum antiserum, and egg albumin antiserum.

TABLE 16

COMPLEMENT-FIXATION TEST WITH SWINE SERUM

Dilution of Antigen	Swine Serum Antiserum
0.05 c.c.	—
0.025 c.c.	—
0.0125 c.c.	—
0.00625 c.c.	—
0.000312 c.c.	—
0.00126 c.c.	—
0.00078 c.c.	—
0.00039 c.c.	—
0.000195 c.c.	—
0.000097 c.c.	—
0.000048 c.c.	—
0.000024 c.c.	—
0.000012 c.c.	Trace +
0.000006 c.c.	Partial +
0.000003 c.c.	Almost +
No antigen	+

Complete hemolysis with normal rabbit serum, ox tendon mucin antiserum, ox submaxillary mucin antiserum, swine stomach mucin antiserum, ox serum antiserum, and egg albumin antiserum.

TABLE 17

COMPLEMENT-FIXATION TEST WITH CRYSTALIZED EGG ALBUMIN

Dilution of Antigen 1 Percent Solution	Egg Albumin Antiserum
0.5 c.c.	Trace +
0.25 c.c.	—
0.125 c.c.	—
0.0625 c.c.	—
0.03125 c.c.	—
0.0156 c.c.	—
0.0078 c.c.	—
0.0039 c.c.	—
0.00195 c.c.	—
0.00097 c.c.	—
0.00048 c.c.	—
0.00024 c.c.	—
0.00012 c.c.	Partial +
0.00006 c.c.	Partial +
0.00003 c.c.	Almost +
No antigen	+

Complete hemolysis with normal rabbit serum, ox tendon mucin antiserum, ox submaxillary mucin antiserum, pig stomach mucin antiserum, ox serum antiserum, and pig serum antiserum.

other compound proteins as well as simple proteins modified by the addition of various substances.

Each mucin gives rise to an antiserum that reacts with itself in comparatively high dilutions; with the blood serum of the same species to a less degree; and with the other mucins almost as well as with the blood serum. The reaction with the other mucins is independent of species and the antiserum does not react with the blood serum of the different species.

The present study does not throw any light on the nature of the union of the protein and carbohydrate components of the glycoproteins, excepting that it demonstrates that the antigenic properties of this class of compound proteins are different from the antigenic properties of another common class of compound proteins, the "nucleoproteins," which as such are said to produce specific antibodies for themselves. While the antibodies produced by the mucins do react with the blood serum of the same species, still there are very marked quantitative differences in favor of the homologous mucin. In so far as the antibodies produced by one mucin react with other mucins from a different species as well as from the same species, support is given to the theory that specificity depends on the chemical nature of the antigenic protein rather than its biological origin.